



Factor Xa and thrombin evoke additive calcium and proinflammatory responses in endothelial cells subjected to coagulation

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Abstract

Endothelial cells react to factor Xa and thrombin by proinflammatory responses. It is unclear how these cells respond under physiological conditions, where the serine proteases factor VIIa, factor Xa and thrombin are all simultaneously generated, as in tissue factor-driven blood coagulation. We studied the Ca^{2+} signaling and downstream release of interleukins (ILs), induced by these proteases in monolayers of human umbilical vein endothelial cells. In single cells, factor Xa, but not factor VIIa, complexed with tissue factor, evoked a greatly delayed, oscillatory Ca^{2+} response, which relied on its catalytic activity and resembled that of SLIGRL, a peptide specifically activating the protease-activated receptor 2 (PAR2). Thrombin even at low concentrations evoked a rapid, mostly non-oscillating Ca^{2+} response through activation of PAR1, which reinforced the factor Xa response. The additive Ca^{2+} signals persisted, when factor X and prothrombin were activated in situ, or in the presence of plasma that was triggered to coagulate with tissue factor. Further, thrombin reinforced the factor Xa-induced production of IL-8, but not of IL-6. Both interleukins were produced in the presence of coagulating plasma. In conclusion, under coagulant conditions, factor Xa and thrombin appear to contribute in different and additive ways to the Ca^{2+} -mobilizing and proinflammatory reactions of endothelial cells. These data provide first evidence that these serine proteases trigger distinct signaling modules in endothelium that is activated by plasma coagulation.

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1. Introduction

Coagulation, i.e., formation of fibrin, is initiated by complex formation of tissue factor with the protease factor VIIa and is propagated by proteolytic activity of two other proteases, factor Xa and thrombin [1]. Factor X is activated by the tenase complex in the presence of phospholipids to form factor Xa. The latter forms with cofactor Va and phospholipids the prothrombinase complex, which then cleaves prothrombin into thrombin. Activated cells are considered to provide the procoagulant phospholipid surface. There is broad evidence

that the coagulation proteases, factor VIIa, factor Xa and thrombin, once activated, also evoke a variety of responses in cells from the vascular system. For instance, in endothelial and other cells, both proliferative and acute inflammatory effects of the activated coagulation factors have been described [2–4]. In endothelial cells, the proinflammatory response induced by factor Xa consists of cytokine production and expression of adhesion molecules [5,6].

Endothelial cells contain at their surface a number of protease-activated receptors (PARs), which, following cleavage, can couple to signaling G proteins. There is general agreement that thrombin activates Gq-coupled PAR1 and PAR4 on endothelial cells [7–9], but by which receptors factor Xa signals are still unclear. Earlier, it was thought that factor Xa acts through the elusive effector cell protease receptor 1 (EPR1) [10,11], but the existence of this receptor is now questionable

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[12]. More recently, it was proposed that the trypsin substrate PAR2 acts as receptor for factor Xa [13]. However, expression studies with cell lines indicate that factor Xa can also signal via PAR1 and PAR4, while knockout mouse models suggest that PAR1 along with PAR2 contributes to factor Xa-induced activation of endothelial cells [9]. Recent evidence also suggests co-activity of the coagulation proteases, e.g., the ternary complex of tissue factor and factor VIIa and factor Xa is considered to be more potent than its separate components on cellular activation, when formed on the surface of tissue factor-expressing cells [14,15].

In vivo, endothelial cells subjected to conditions of hemostasis or thrombosis are faced with the combination of all coagulation proteases and not with single factors. However, only very little is known of the endothelial responses under conditions of coagulation, when all these proteases are simultaneously generated. In this paper, we present single cell data, showing that factor Xa and thrombin evoke distinct and additive Ca^{2+} responses in confluent monolayers of native endothelial cells. These different Ca^{2+} responses were accompanied by additive effects on cytokine release and were maintained in the presence of coagulating plasma. The results thus suggest that different sets of receptors and signaling routes are involved in the endothelial reactions to factor Xa and thrombin generated during coagulation.

2. Materials and methods

2.1. Materials

Hirudin was obtained from BASF (Ludwigshafen, Germany). Fura-2 and Fura-red acetoxymethyl esters were from Molecular Probes (Leiden, The Netherlands); annexin A5 was from Nexins Research (Hoeven, The Netherlands); SLIGRL and SFLLRN from Bachem (Bubendorf, Switzerland). Chromogenic factor Xa substrate (Pefachrome Xa) was from Pentapharm (Basel, Switzerland), and thrombin-cleavable substrate (S2238) from KabiVitrum (Stockholm, Sweden).

Recombinant human tissue factor containing the membrane-spanning domain (Innovin) was from Dade Behring (Marburg, Germany). Soluble, 219 residues tissue factor was a generous gift from Henogen (Gosselies, Belgium). Human prothrombin (factor II) and bovine factor Va were purified, as described before [16]; human thrombin was from Sigma (St. Louis, MO, USA). Factor Xa was purified from bovine and human plasma as described [17] and activated with Russell's viper venom [18]. Factor Xa activity was measured by the ability to hydrolyse Pefachrome Xa; purity was controlled by SDS-PAGE and staining with Coomassie brilliant blue. Modified bovine factor Xa, lacking the γ -carboxyglutamic acid (Gla) residues (factor Xa-gla), was a kind gift from Drs. J. Rosing and G. Tans (Dept. of Biochemistry, University of Maastricht) and was prepared as described elsewhere [19]. The concentration of factor Xa-gla was calculated from this activity toward Pefachrome Xa, assuming identical amidolytic activity to factor Xa. Active site-inhibited factor Xa was obtained by incubation of native factor Xa with dansyl-glutamylglycyl-L-arginine (DEGR) chloromethyl ketone (Calbiochem, La Jolla CA, USA), as described [20]. Antithrombin was obtained from bovine plasma and purified to homogeneity as described by others [21]. Other materials were obtained from Sigma (St. Louis, MO, USA).

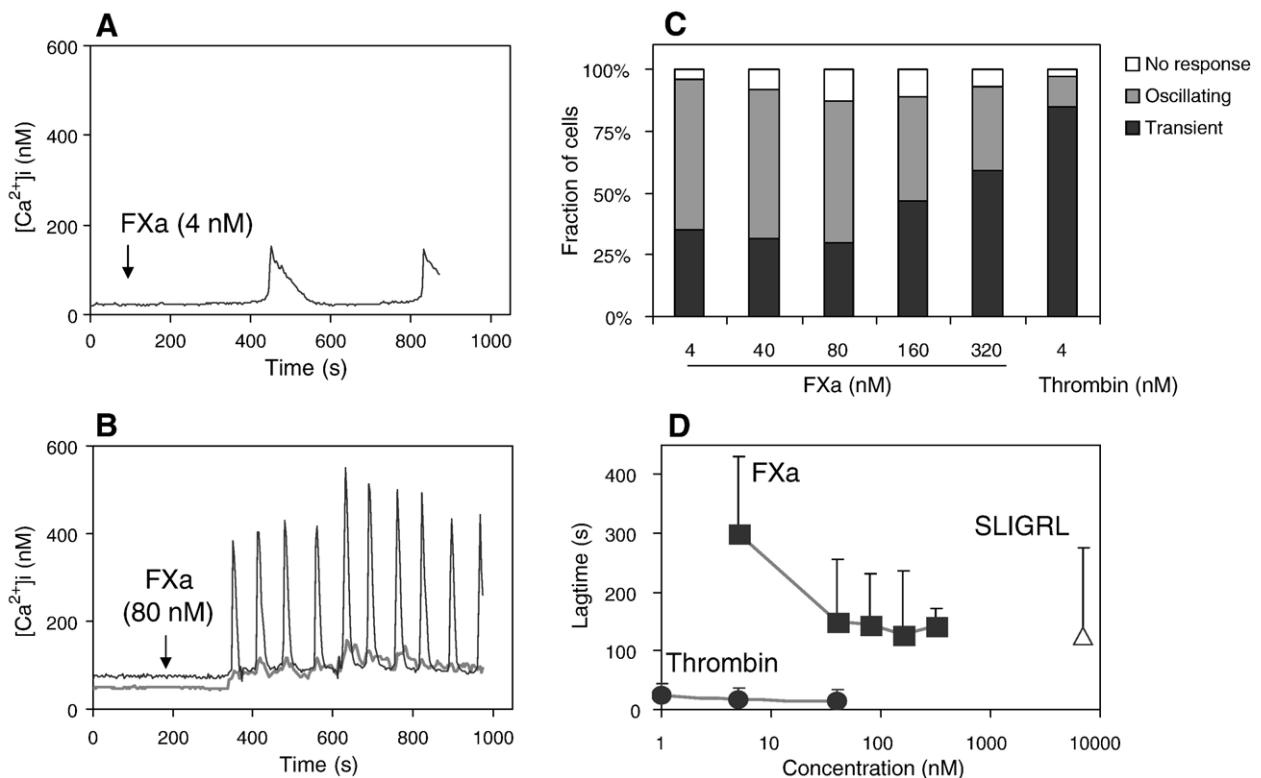


Fig. 1. Characteristics of factor Xa-induced Ca^{2+} responses in HUVEC. Confluent monolayers of Fura-2-loaded HUVEC were stimulated with factor Xa (FXa, 4–320 nM), thrombin (1–40 nM) or SLIGRL (10 μM) in HEPES buffer pH 7.4 containing 1.8 mM CaCl_2 at 37 °C. (A, B) Changes in $[\text{Ca}^{2+}]_i$ in response to 4 or 80 nM FXa. Shown are traces of a representative cell with oscillations (black), and averaged traces of 27 cells (grey). (C) Fractions of cells responding to 4–320 nM FXa (19–104 cells) or 4 nM thrombin: no Ca^{2+} response (white), oscillating Ca^{2+} response (grey), or non-oscillating prolonged response (black). (D) Lagtime before first $[\text{Ca}^{2+}]_i$ increase after stimulation with thrombin, factor Xa or SLIGRL (mean \pm S.E.M., 90–104 cells). Data are from at least three independent experiments.

2.2. Endothelial cell culturing and stimulation

HUVEC were isolated from fresh umbilical cords by treatment with collagenase type I (Sigma) and were grown in RPMI 1640 medium as described [5]. Cells of passages 1 through 4 were seeded on coverslips or culture plates at a density of $5 \times 10^4/\text{cm}^2$ and grown to confluency. For real-time $[\text{Ca}^{2+}]_i$ measurements, cells were grown on gelatine-coated glass coverslips [22]. Briefly, coverslips were activated with 0.5% (w/v) glutaraldehyde in phosphate-buffered saline (PBS) and then coated with 1% (w/v) gelatine (Difco, Detroit, USA) in PBS. After a wash step, the gelatine on coverslip was treated with 0.5% glutaraldehyde in PBS for 30 min, washed twice, and HUVEC were seeded. The endothelial cells were cultured for 2–3 days to confluence in air containing 5% CO_2 at 37 °C. For interleukin and coagulation measurements, the HUVEC were grown on fibronectin-coated 96-well flat-bottom tissue culture plates in RPMI 1640 medium [5]. Cells on coverslips of wells were stimulated with indicated concentrations of factor Xa, thrombin or other (ant)agonists at 37 °C. In some experiments, the cells were incubated with bacterial-free human plasma and then stimulated with CaCl_2 and tissue factor.

2.3. Measurement of cytosolic Ca^{2+} concentration

Confluent monolayers of HUVEC on coverslip were loaded with fluorescent Ca^{2+} probe, basically as described [23]. The cells were washed twice with HEPES buffer pH 7.4, consisting of 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM HEPES, 5 mM glucose, 4 mM glutamine, 0.25% (w/v) bovine serum albumin (BSA) and 1.8 mM CaCl_2 . For most experiments, the cells were then loaded with 1 μM Fura-2 acetoxymethyl ester in HEPES buffer containing 1% BSA for 30 min at 37 °C. For experiments with coagulating plasma, the cells were loaded with 10 μM Fura-red acetoxymethyl ester in the same buffer for 20 min at 37 °C. After loading with fluorescent probe, the cells were incubated with HEPES buffer for 15 min to allow complete de-esterification of the dye.

Coverslips with loaded cells were mounted in a 1 ml thermostated (37 °C) open chamber placed on a Nikon Diaphot or Olympus inverted microscope. Factor Xa, thrombin and other agonists were added to the chamber in volumes of 100–200 μl in HEPES buffer pH 7.4. All concentrations given are final. Changes in Fura-2 fluorescence in individual cells were measured by calibrated ratio fluorometry at 340 and 380-nm excitation, using a camera-based Quanticell video imaging system (Visitech, Sunderland, UK) [24]. Changes in Fura-red fluorescence were measured by ratio fluorometry using a camera-based TillVision imaging system (Till Photonics, Gräfelfing, Germany). Fluorescence at excitation at 463 and 472 nm was detected at 660 nm, and ratio values were obtained. In the latter case, no calibration was applied, since the clot formation interfered with the fluorescence detection.

2.4. Coagulation measurements

Blood, drawn from healthy donors, was collected in 1/10 volume of 129 mM trisodium citrate. Platelet-poor plasma (PPP) was prepared by centrifugation [25]. Generation of factor Xa and clotting times were measured as described [16]. Thrombin generation was measured in a continuous assay, as described [25].

2.5. Measurement of interleukins

To measure interleukins (IL), supernatants were harvested from HUVEC on culture platelet at the end of incubation periods. Cytokine levels were determined using an earlier published sandwich ELISA for IL-6 and IL-8 [5]. The lower detection limit of the immunoassays was 10 pg/ml. Expression of adhesion molecules on activated HUVEC was detected, as described [5].

2.6. Statistical analysis

Data are shown as means \pm S.E.M. Statistical significance was determined using ANOVA and Bonferoni post-hoc analysis using Prism 4 software (Graph Pad Inc.). Data were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Factor Xa and thrombin evoke distinct Ca^{2+} responses in single endothelial cells

In populations of suspended endothelial cells, purified factor Xa causes phospholipase C activation [9,13] and a small, transient Ca^{2+} mobilization [26]. We aimed to study the factor Xa-induced Ca^{2+} signaling at more physiological conditions, by using confluent monolayers of adherent human endothelial cells. Early passage HUVEC, grown to confluency on gelatine-coated coverslips, were loaded with the fluorescence ratio probe Fura-2, and changes in fluorescence were monitored per individual cell by microscopic fluorescence video imaging [23]. In the majority of cells (61%) in monolayer, addition of >4 nM bovine factor Xa elicited a strongly delayed, oscillatory change in $[\text{Ca}^{2+}]_i$ (Fig. 1A). Other responsive cells showed a delayed, single transient increase in $[\text{Ca}^{2+}]_i$. At a concentration of 80 nM factor Xa, the oscillation pattern was often regular in appearance (57/100 cells, Fig. 1B).

Dose–response curves indicated that the average spiking frequency increased from 0.10 to 0.27 and 0.35 min^{-1} at 5, 40 and 80 nM factor Xa, respectively. The fraction of cells with oscillations decreased from 61% to 34%, when factor Xa was given above physiological concentrations of 160–

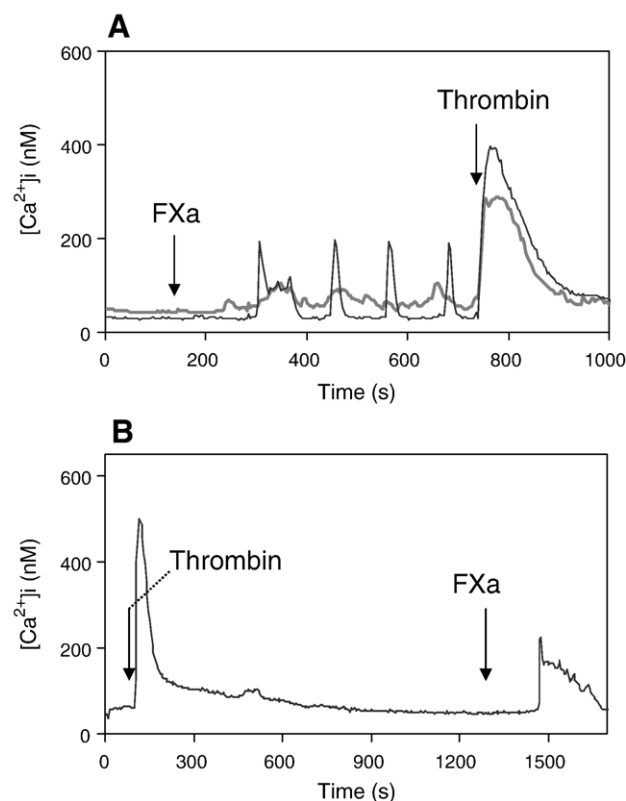


Fig. 2. Additional Ca^{2+} responses with factor Xa and thrombin. Fura-2-loaded HUVEC were stimulated as described for Fig. 1. (A, B) Traces of $[\text{Ca}^{2+}]_i$ upon stimulation with FXa (40 nM) and thrombin (10 nM), as indicated. Shown are traces of a single cell (black) and averaged traces of 25 cells (grey). Data are representative for 3 or more independent experiments.

320 nM (Fig. 1C). Typically, at these high factor Xa concentrations, the cell fraction with oscillatory responses again increased to 72%, when CaCl_2 was omitted from the incubation medium.

Remarkably, all factor Xa-stimulated cells showed a long lagtime of several minutes before onset of the Ca^{2+} response which then continued for a long time (Fig. 1D). Although quite variable from cell to cell, the averaged lagtime reduced with the factor Xa concentration, reaching a plateau level of 150 s at >40 nM factor Xa. Because the Ca^{2+} responses of individual HUVEC were asynchronous, the averaged response of all cells in the monolayer appeared as a delayed, transient $[\text{Ca}^{2+}]_i$ increase. In three experiments, the averaged maximal increase at 40 nM factor Xa amounted to 95 ± 20 nM $[\text{Ca}^{2+}]_i$. Bovine and human factor Xa caused similar Ca^{2+} response patterns.

Factor Xa-induced activation of endothelial phospholipase C is supposed to be mediated by PAR2 and possibly PAR1, while thrombin can signal via PAR1 and PAR4 [9]. This suggests that factor Xa and thrombin can have partially overlapping effects on Ca^{2+} signaling. We found that, in HUVEC, thrombin (1–40 nM) caused a quite different type of Ca^{2+} response, consisting of rapid, broad Ca^{2+} transient (Fig. 2). Only a minority of about 10% of the cells showed signs of multiple Ca^{2+} oscillations, in this case with a high spiking frequency of 1.8 min^{-1} (Fig. 1C). In marked contrast to factor Xa, all cells responded to low and high doses of thrombin by an almost immediate increase in $[\text{Ca}^{2+}]_i$ (Fig.

1D). At 4 nM thrombin, the lagtime of 126/132 cells was 10–15 s, which was the time of diffusion of the agonist to the cells. SFLLRN is able to activate PAR1 and PAR2 but with different affinities (PAR-1 being 16 times more affine than PAR2) as shown in 2001 by Maryanoff et al. [27]. Like thrombin, the PAR1 agonist SFLLRN caused an immediate transient Ca^{2+} signal (not shown). Thrombin also evoked an immediate, transient $[\text{Ca}^{2+}]_i$ increase when applied after factor Xa (Fig. 2A). On the other hand, after stimulation with thrombin, most cells still responded to factor Xa by a delayed Ca^{2+} signal (Fig. 2B).

Similarly to factor Xa, the specific PAR2 agonist SLIGRL, at a high dose of 10 μM , evoked a greatly delayed Ca^{2+} response in most HUVEC (Fig. 1D). Also in this case, responses were oscillatory in 68% (50/73) of the cells. Several other observations pointed to high similarity of the factor Xa- and SLIGRL-induced responses. For instance, in some cells, factor Xa (Fig. 3A) and SLIGRL (Fig. 3C) evoked a multi-spike $[\text{Ca}^{2+}]_i$ transient that was followed by single-spike oscillations. Second, addition of SLIGRL to cells which were preincubated with a moderate dose of factor Xa did not alter the oscillating Ca^{2+} response pattern (Fig. 3B). Conversely, factor Xa did not modulate the response to SLIGRL (Fig. 3C). Further, in cells preactivated with a high dose of factor Xa, SLIGRL caused only little Ca^{2+} spiking, while thrombin induced a high $[\text{Ca}^{2+}]_i$ increase (Fig. 3D). Together, this indicates that activated PAR2 plays a major if not sole role in the factor Xa-induced Ca^{2+} response of HUVEC.

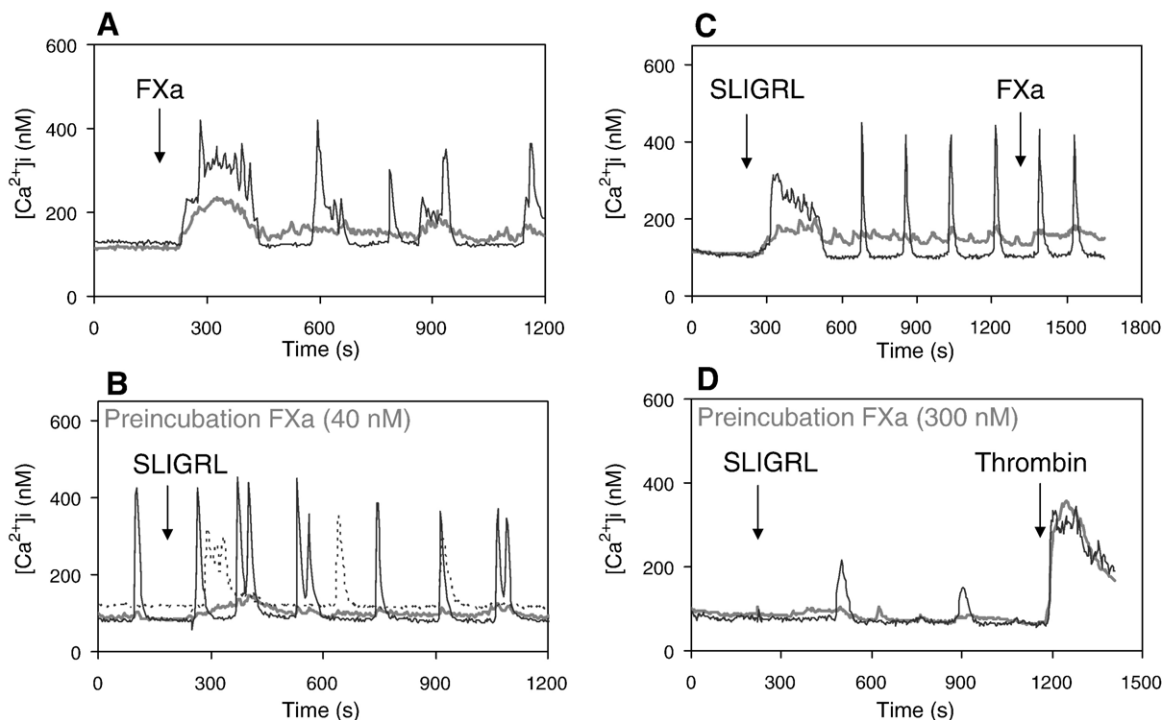


Fig. 3. Similarity of Ca^{2+} responses with factor Xa and PAR2 agonist SLIGRL. Fura-2-loaded HUVEC were preincubated with FXa (40–300 nM) for 20 min, where indicated. Subsequently, the cells were stimulated with FXa (40 nM), SLIGRL (10 μM) or thrombin (4 nM), and Ca^{2+} responses were monitored. Shown are traces of a representative, single cell (black and dashed lines) and averaged traces (grey). Panels give typical, oscillating Ca^{2+} responses observed with FXa (A) or SLIGRL (C). (B) Continuation of panel A; stimulation of the same cells with SLIGRL after 20 min. (D) Cells pretreated with FXa (300 nM) for 20 min were stimulated with SLIGRL and thrombin.

3.2. Factor Xa-induced Ca^{2+} response relies on its catalytic activity and is not enhanced by factor VIIa

The structural requirements of factor Xa for causing the oscillatory changes in Ca^{2+} were investigated. Treatment of factor Xa with antithrombin – which is a physiological inhibitor of both factor Xa and thrombin – completely abolished the Ca^{2+} signal in HUVEC (Fig. 4A). In contrast, treatment with hirudin – which only inhibits thrombin activity – did not influence the factor Xa-induced Ca^{2+} response. A modified form of factor Xa was tested, lacking the Gla residues responsible for Ca^{2+} -dependent binding, e.g., to phospholipids [1]. While this form did not evoke a Ca^{2+} signal, intriguingly, it caused immediate oscillations when replaced by unmodified factor Xa (Fig. 4B). However, the factor Xa-induced activation did not rely on binding to procoagulant phospholipids, since it was not influenced by phosphatidylserine-binding annexin A5. To investigate the importance of the proteolytic activity, factor Xa was treated with DEGR-chloromethyl ketone, which inactivates its catalytic site. The loss of protease activity was verified by amidolytic measurement. Active site-inhibited factor Xa was non-responsive to HUVEC, but normal Ca^{2+} responses occurred when it was replaced by unmodified factor Xa (Fig. 4C). Further, post-addition of DEGR-chloromethyl ketone immediately blocked all factor Xa effects (Fig. 4D).

In conclusion, these results indicate that the continuous presence of the active (proteolytic) site and Gla domains are needed for factor Xa to induce Ca^{2+} oscillations, likely via proteolytic receptor cleavage.

In transfected cells where membrane-bound tissue factor and factor VIIa are co-localized on the cell surface, it has been described that the tissue factor–factor VIIa complex facilitates the factor Xa-dependent signaling [14]. We used two forms of tissue factor to study its Ca^{2+} -mobilizing effect in endothelial cells: a recombinant soluble tissue factor fragment, which binds to factor VII(a) with low affinity, and recombinant longer chain tissue factor (Innovin), containing the membrane-spanning domain with a much higher affinity for factor VII(a). First, the doses of soluble (100 nM) and of long-chain (10 pM) tissue factor were established that gave clotting times in recalcified phospholipid-containing plasma of about 120 s. The HUVEC were then treated at these tissue factor concentrations in the presence of equimolar factor VIIa. Neither of the tissue factor–factor VIIa complexes was able to cause a significant Ca^{2+} response (Fig. 5A).

Subsequently, we determined the effect of combined addition of (soluble or membrane-bound) tissue factor, factor VIIa and factor Xa. This resulted in Ca^{2+} responses that were similar in magnitude (Fig. 5B) and shape (not shown, but see below) as the responses evoked by factor Xa alone. In a next

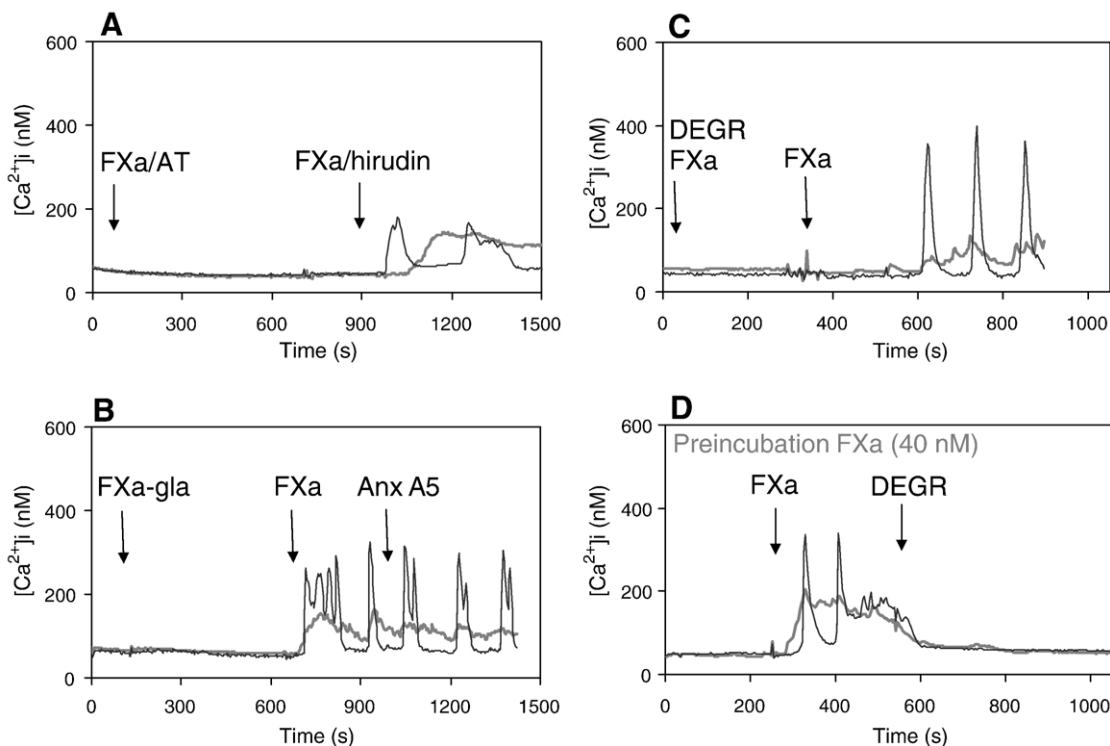


Fig. 4. Requirement of catalytic activity and Gla domain for factor Xa-induced Ca^{2+} responses. Fura-2-loaded HUVEC were used for Ca^{2+} measurements, as described for Fig. 1. (A) Cells were treated with a mixture of 40 nM FXa and 160 nM antithrombin (AT), which was later replaced by a mixture of 40 nM FXa and 5 U/ml hirudin. (B) Cells were treated with 80 nM FXa lacking Gla residues (FXa-gla), which was later replaced by 80 nM FXa, after which 15 $\mu\text{g}/\text{ml}$ annexin A5 was added. (C) Cells were treated with 80 nM active site-inhibited FXa (DEGR-FXa), which was later replaced by 80 nM FXa. (D) Cells were preactivated with low 10 nM FXa for 20 min, after which 80 nM FXa and 10 μM DEGR-chloromethyl ketone were added. Representative traces are shown of a single cell (black) and averaged of 15–25 cells (2–3 experiments) (grey).

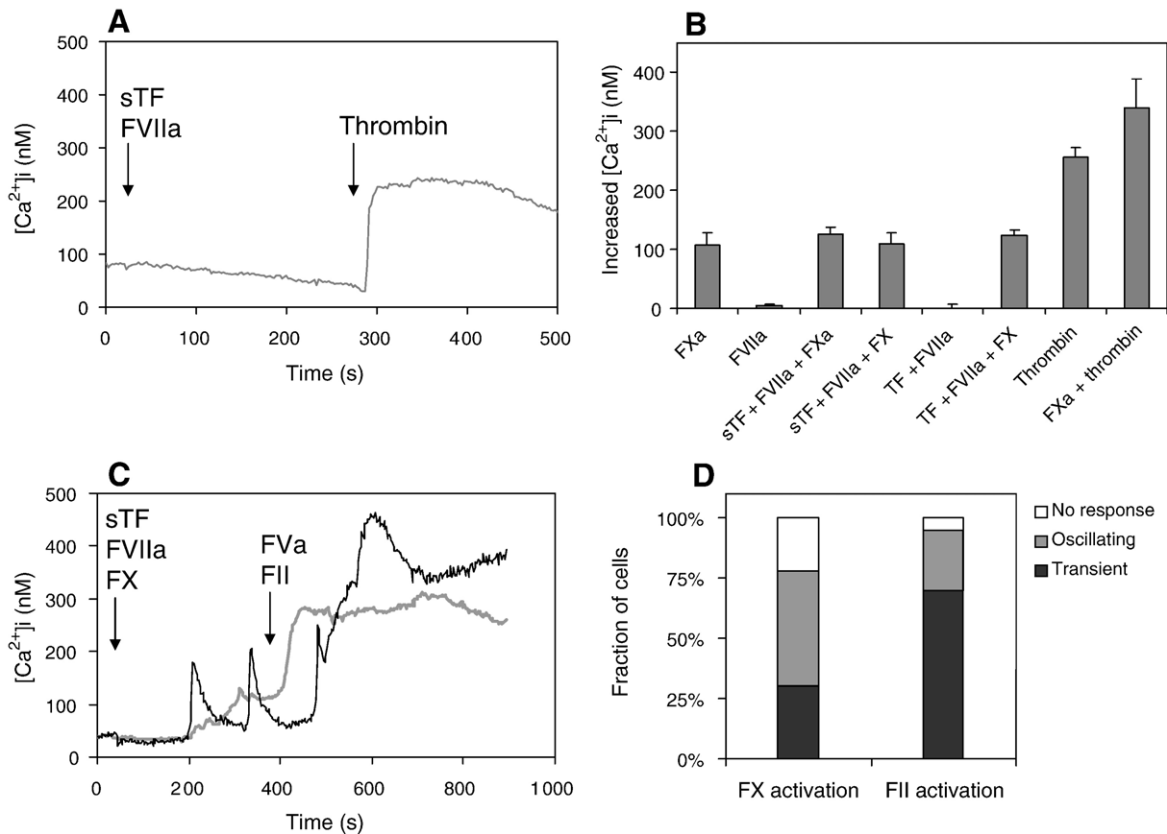


Fig. 5. Additional effects of in situ generated factor Xa and thrombin on Ca^{2+} signaling. (A) Averaged $[Ca^{2+}]_i$ trace of Fura-2-loaded HUVEC incubated with 100 nM soluble tissue factor (sTF), 100 nM factor VIIa (FVIIa), followed by 10 nM thrombin. (B) Maximal increases in $[Ca^{2+}]_i$ in HUVEC are given induced by the indicated combinations of 40 nM FXa or FX, 100 nM FVIIa, 100 nM sTF, and 10 nM thrombin; alternatively $[Ca^{2+}]_i$ increases with 10 pM full-length TF in combination with 10 pM FVIIa. Data are from averaged traces (mean values \pm S.E.M., $n=3-9$ experiments). (C, D) Calcium responses due to in situ activation of FX and prothrombin (FII). HUVEC were incubated with 100 nM sTF, 100 nM FVIIa and 40 nM FX. Subsequently, thrombin generation was induced with 0.1 nM FVa and 10 nM FII. Traces are given of a representative cell (black) and averaged of 32 cells (grey). (D) Fractions of cells responding to in situ FX and FII activation: no Ca^{2+} response (white), oscillating Ca^{2+} response (grey), or non-oscillating prolonged response (black).

step, factor X was activated in situ by adding to the HUVEC a mixture of soluble tissue factor, factor VIIa and factor X. Parallel factor Xa generation measurements indicated that, at the cofactor concentrations used (see Fig. 5B), the conversion of factor X into Xa was complete for 91% in 30 s. Similarly to factor Xa itself, the mixtures provoked delayed, oscillatory Ca^{2+} responses (Fig. 5C), which on average had a mean size of 110–123 nM, i.e., similar to the mean response of added factor Xa (Fig. 5B). Importantly, subsequent addition of factor Va and prothrombin (factor II) to provoke thrombin (factor IIa) generation resulted in a second, much higher Ca^{2+} response in all cells. Parallel measurements of thrombin generation indicated that, in 60 s, 99% of all prothrombin was converted into thrombin.

Similarly as observed with factor Xa or thrombin alone, a much higher fraction of the cells displayed oscillatory changes in $[Ca^{2+}]_i$ during the period of in situ factor X activation than during the next period of prothrombin activation (Fig. 5D). Together, these results thus show that HUVEC monolayers respond in a similarly way to in situ formed factor Xa and thrombin as to addition of the preactivated factors. Furthermore, the factor Xa-induced response is not enhanced by tissue factor and factor VIIa.

3.3. Factor Xa and thrombin differently evoke cytokine release in endothelial cells

The distinct factor Xa- and thrombin-induced Ca^{2+} responses in endothelial monolayers point to additive effects of these coagulation proteases on cell activation. This was further investigated by measuring downstream activation events [28], i.e., expression of the proinflammatory cytokines IL-6 and IL-8 [5]. $TNF\alpha$ was used as a positive control, giving high levels of interleukin production. In the HUVEC monolayers, factor Xa but not thrombin evoked a two-fold increase in IL-6 production (Fig. 6A). On the other hand, factor Xa and thrombin potently induced IL-8 production; thrombin further increased the effect of factor Xa (Fig. 6B). Thus, both proteases appear to be involved in IL-8 production, but factor Xa in IL-6 production.

3.4. Endothelial cells treated with coagulating plasma respond to factor Xa and thrombin

The physiological significance of these findings was assessed by subjecting HUVEC to coagulating human plasma. Because plasma is highly fluorescent at the Fura-2 340/380-nm

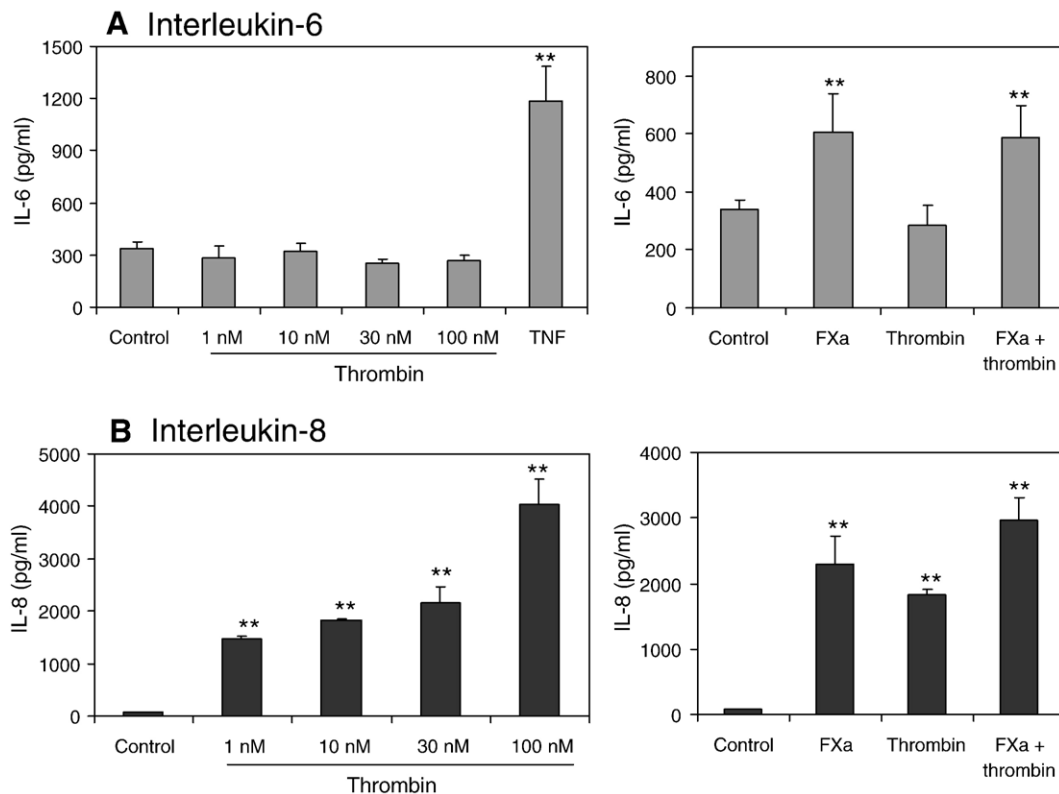


Fig. 6. Variable effects of factor Xa and thrombin on interleukin production by HUVEC. HUVEC in culture plates were treated with control vehicle, thrombin (1–100 nM), factor Xa (40 nM) or tumor necrosis factor- α (TNF, 20 ng/ml). Supernatants were collected from cells after 20 h of treatment, and assayed for IL-6 and IL-8 production. Data are mean values \pm S.E.M. of 4 or more experiments. ** $P < 0.05$ compared to control.

excitation wavelengths, $[Ca^{2+}]_i$ measurements were performed with HUVEC that were loaded with the longer wavelength probe Fura-red, which also shows a small but significant shift in fluorescence ratio upon Ca^{2+} binding. Calibrated control experiments with Fura-red-loaded HUVEC indicated that factor Xa in the presence of HEPES buffer caused the normal oscillating Ca^{2+} responses (250-nM peak levels), although in total, only 58% of the cells were responsive (data not shown), likely due to the high concentration of Fura-red needed for fluorescence detection.

The Fura-red-loaded HUVEC were incubated with plasma that was triggered to coagulate with soluble tissue factor and $CaCl_2$. Control measurements of factor Xa and thrombin generation in plasma indicated that 0.5 μ M soluble tissue factor/ $CaCl_2$ induced after 1 min levels of 250 nM and 9 nM, and after 2 min levels of 150 nM and 9 nM of thrombin and factor Xa, respectively. In the presence of 100 U/ml hirudin, thrombin generation was abolished, while factor Xa still peaked at about 9 nM (see also [29]). Tissue factor addition evoked in many cells an initial transient Ca^{2+} signal, which was followed by one or more Ca^{2+} oscillations (Fig. 7A). Importantly, in plasma preincubated with hirudin to block the generation of thrombin but not factor Xa, all responding cells showed oscillatory $[Ca^{2+}]_i$ changes (Fig. 7A, B). Again, only part of the Fura-red-loaded cells was responsive, with about 45% showing no $[Ca^{2+}]_i$ increase.

Measurements of cytokine production indicated that soluble tissue factor dose-dependently increased the production of both IL-6 and IL-8 (Fig. 8). Taken together, these results indicate that the HUVEC in contact with coagulating plasma responded to both generated factor Xa and thrombin by specific patterns of Ca^{2+} responses and interleukin release.

4. Discussion

The present results show that factor Xa, similarly as the PAR2-specific agonist SLIGRL, causes greatly delayed, oscillatory Ca^{2+} responses in HUVEC with lag times of two or more minutes, depending on the factor Xa concentration. Such delayed, oscillatory responses have not yet been described for endothelial cells triggered with coagulation factors, and point to a unique receptor-activating signaling pathway. It has not yet been identified, likely because others have concentrated on measurements in suspensions of non-adherent endothelial cells, e.g., determining factor Xa-induced phospholipase C activity [9,13,26] and/or Ca^{2+} signaling [7,26]. It is also conceivable that such cells are already in an activated state by being in suspension. Typically, the delayed responses in endothelium are also distinct from the factor VIIa- and factor Xa-induced oscillatory Ca^{2+} responses in Madin-Darby canine kidney cells, which present a very short delay time and are

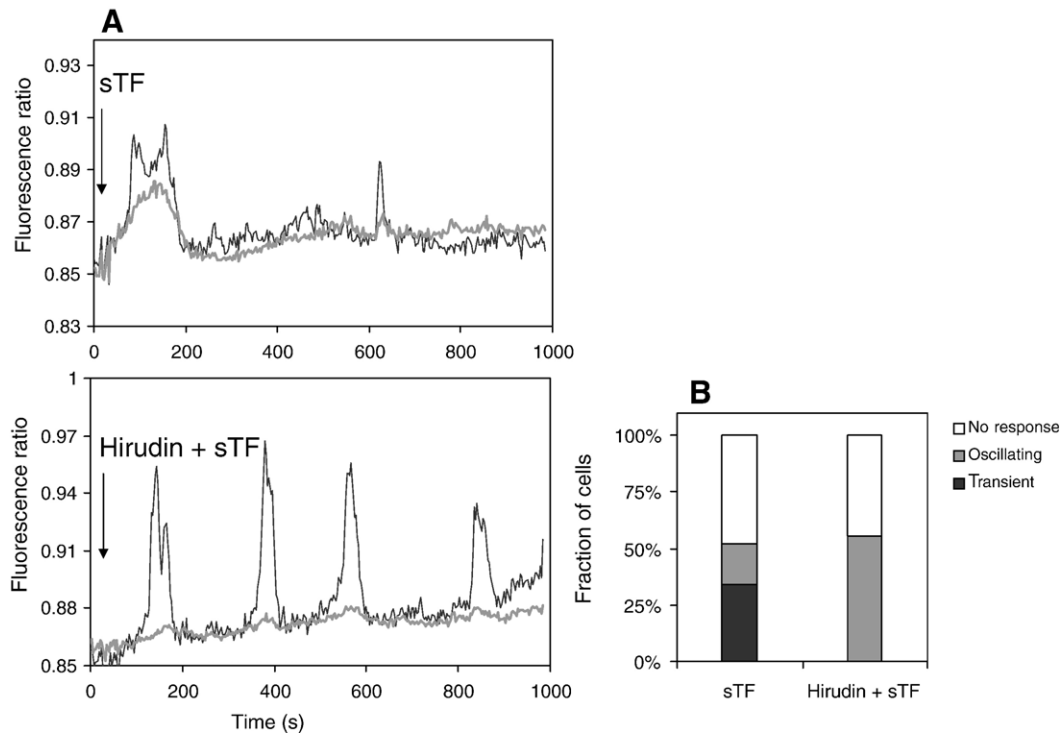


Fig. 7. Effects of factor Xa and thrombin generation in plasma on Ca^{2+} signaling in HUVEC. (A) Fura-red-loaded HUVEC were incubated with human plasma, which was triggered with $0.15 \mu\text{M}$ soluble tissue factor (sTF) and 16.6 mM CaCl_2 . Plasma was preincubated with 100 U/ml hirudin to block thrombin activity, where indicated. Fluorescence ratio traces of Ca^{2+} responses given of single cells and populations of cells ($n=10$) are representative for 3–4 experiments. (B) Fractions of cells responding sTF: no Ca^{2+} response (white), oscillating Ca^{2+} response (grey), or non-oscillating prolonged response (black). Control runs showed that hirudin alone did not induce Ca^{2+} signaling (not shown).

thought to be triggered via a receptor analogous to PAR1 or PAR2 [30]. The mechanism underlying the delayed response in HUVEC is unclear. However, a clue comes from the observation that pretreatment with a Gla domain-lacking factor Xa greatly shortens this lag time. This may imply that factor Xa-induced activation occurs after slow binding to the receptor (PAR2) and/or via slow transactivation of another receptor (see below). However, other explanations like delayed intracellular reactions cannot be ruled out.

After recognition that the gene of the elusive factor Xa receptor, EPR1 [10], in fact encodes for a protein regulating apoptosis [12], it has been a matter of debate which receptor(s) mediate factor Xa-induced effects on vascular cells. In agreement with previous data of factor Xa-induced cytokine production [5], we have not found evidence for involvement of EPR1 in the Ca^{2+} responses of HUVEC. For instance, EPR1-derived peptides, designed to interact with factor Xa, were unable to influence the factor Xa responses (N. Senden, unpublished data). Recent findings with knockout mice imply that in murine endothelial cells both PAR1 and PAR2 can contribute to factor Xa effects [9]. The same conclusion was drawn for vascular cells from receptor desensitization studies with thrombin and peptide agonists of PAR1 and PAR2 [31]. In HeLa cells, it was concluded that factor Xa similarly to thrombin can elicit NF-kappa B activation but in a delayed way [32]. Our findings with human endothelial cells indicate that delayed

signaling is a characteristic effect of factor Xa and the PAR2-specific peptide agonist SLIGRL. Since this peptide does not enhance the Ca^{2+} signaling activity of factor Xa, it appears that PAR2 acts as the main functional factor Xa receptor in HUVEC. Another typical observation is that tissue factor and factor VIIa do not further enhance the Ca^{2+} signal with factor Xa. This indicates that ternary complex formation of factor Xa with tissue factor and factor VIIa [14] is not a major determinant of factor Xa-induced signaling in the HUVEC. Others have suggested that endothelial PAR2 may be activated directly by tissue factor/factor VIIa and indirectly by tissue factor/factor VIIa generated factor Xa [13]. The present Ca^{2+} signaling patterns indicate that in resting HUVEC the latter pathway is by far predominant. The slow kinetics of the Ca^{2+} responses with factor Xa or SLIGRL in this respect suggest that, if transactivation occurs, factor Xa-activated PAR2 signals to activate and cleave PAR1, rather than the other way around as previously suggested [8].

The present data add well to earlier work showing that exposure of HUVEC to factor Xa stimulates the production of proinflammatory cytokines including IL-6 and IL-8, and de novo expression of adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [5,6]. Our work is also in accordance with the work on (micro)vascular endothelial cells, also producing IL-6 and IL-8 in response to PAR2 activation by tryptase or peptides [31,33]. A novel

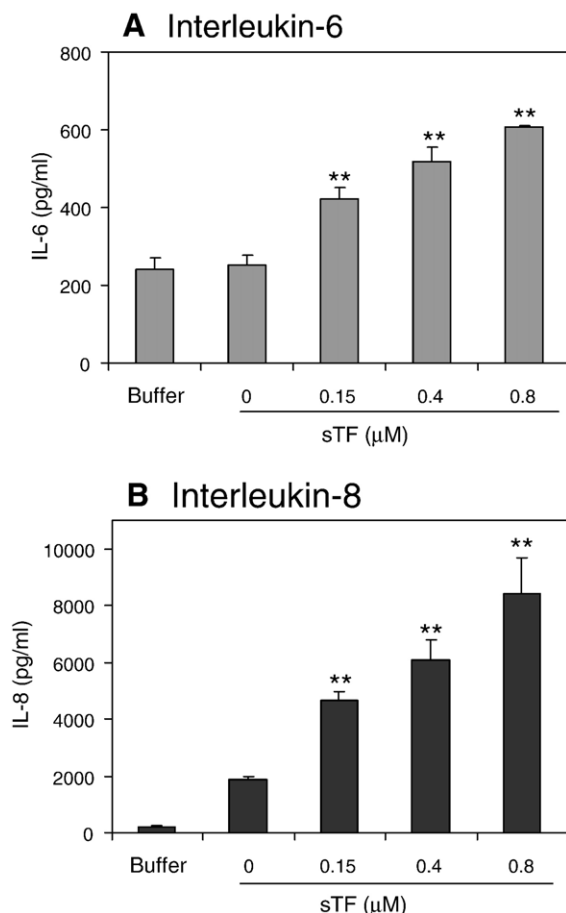


Fig. 8. Effect of coagulating plasma on interleukin production by HUVEC. HUVEC in culture plates were incubated with HEPES buffer or with 100 μ l recalcified plasma in the absence (control) or presence of 0.15–0.8 μ M soluble tissue factor (sTF). After 20 h, supernatants were collected and assayed for IL-6 and IL-8. Data are mean values \pm S.E.M. ($n=16$); ** $P<0.05$ compared to control plasma.

observation however is that only factor Xa but not thrombin evokes IL-6 production. As far as thrombin is concerned, Weinstein et al. [34] have recently discovered that purified thrombin can be contaminated, which need to cautiously interpret results. More specifically, they elegantly showed that recombinant human thrombin did not induce IL-6 release in microglial cells although purified thrombin above 3 U/ml did. In our study, we used a maximum of 10 U/ml (=100 nM) of thrombin not triggering IL-6 release in HUVEC (Fig. 6A) experiments allowing to reasonably discard any thrombin impurity effect. The literature provides indirect support that the factor Xa-inhibiting antithrombin was found to efficiently block IL-6 production in stimulated HUVEC [35]. Together, this indicates that IL-6 formation evoked by coagulating plasma is caused by the generated factor Xa rather than by thrombin. The data show that factor Xa triggers a relatively prolonged series of Ca^{2+} oscillations. Others have proposed that such long-term oscillations have a function in the Ca^{2+} -dependent activation of transcription factors [36]. Since IL-6 gene expression has recently been associated with calcineurin activity [27], such a mechanism

might be triggered by factor Xa, but we do not yet have direct evidence for this.

Another interesting recent observation is that factor Xa-induced IL-8 production in HUVEC is sensitive to PAR1 antagonism [6]. Our results, combined with Weinstein's [34] and Ludeman's results [37], suggested that IL-8 is the product of PAR-1 signalization and IL-6 the product of PAR2. Indeed, thrombin by being the major ligand for PAR-1 and not PAR2 induces IL-8 response but not IL-6 response. On the contrary FXa is ligand for both PAR-1 and 2 and induce both IL-6 and 8 responses.

In summary, we provide first evidence that under coagulant conditions, factor Xa and thrombin contribute in different and additive ways to the Ca^{2+} -mobilizing and proinflammatory reactions of endothelial cells. These data are illustrative for the interactions between the inflammatory and coagulation pathways. They further show that serine proteases, when simultaneously generated in plasma, trigger distinct signaling modules in endothelium, i.e., cells that are naturally exposed to plasma.

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References

- [1] B. Furie, B.C. Furie, The molecular basis of blood coagulation, *Cell* 53 (1988) 505–518.
- [2] G. Cirino, C. Cicala, M.R. Bucci, L. Sorrentino, J.M. Maraganore, S.R. Stone, Thrombin functions as an inflammatory mediator through activation of its receptor, *J. Exp. Med.* 183 (1996) 821–827.
- [3] E. Camerer, J.A. Rottingen, E. Gjernes, K. Larsen, A.H. Skartlien, J.G. Iversen, H. Prydz, Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the *egr-1* gene, *J. Biol. Chem.* 274 (1999) 32225–32233.
- [4] B.H. Koo, K.H. Chung, K.C. Hwang, D.S. Kim, Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2, *FEBS Lett.* 523 (2002) 85–90.
- [5] N.H.M. Senden, T.M.A.A. Jeunhomme, J.W.M. Heemskerk, R. Wagenvoort, C. van't Veer, H.C. Hemker, W.A. Buurman, Factor Xa induces cytokine production and expression of adhesion molecules in human umbilical vein endothelial cells, *J. Immunol.* 161 (1998) 4318–4324.
- [6] G. Busch, I. Seitz, B. Steppich, S. Hess, R. Eckl, A. Schomig, I. Ott, Coagulation factor Xa stimulates interleukin-8 release in endothelial cells and mononuclear leukocytes, *Arterioscler., Thromb., Vasc. Biol.* 25 (2005) 461–466.
- [7] M. Molino, M.J. Woolkalis, J. Reavey-Cantwell, D. Pratico, P. Andrade-Gordon, E.S. Barnathan, L.F. Brass, Endothelial cell thrombin receptors and PAR-2: two protease-activated receptors located in a single cellular environment, *J. Biol. Chem.* 272 (1997) 11133–11141.

- [8] P.J. O'Brien, N. Prevost, M. Molino, M.K. Hollinger, M.J. Woolkalis, D.S. Woulfe, L.F. Brass, Thrombin responses in human endothelial cells: contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1, *J. Biol. Chem.* 275 (2000) 13502–13509.
- [9] E. Camerer, H. Kataoka, M. Kahn, K. Lease, S.R. Coughlin, Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells, *J. Biol. Chem.* 277 (2002) 16081–16087.
- [10] A.C. Nicholson, R.L. Nachman, D.C. Altieri, B.D. Summers, W. Ruf, T.S. Edgington, D.P. Hajjar, Effector cell protease receptor-1 is a vascular receptor for coagulation factor Xa, *J. Biol. Chem.* 271 (1996) 28407–28413.
- [11] G. Cirino, C. Cicala, M. Bucci, L. Sorrentino, G. Ambrosini, G. DeDominicis, D.C. Altieri, Factor Xa as an interface between coagulation and inflammation: molecular mimicry of factor Xa association with effector cell protease receptor-1 induces acute inflammation in vivo, *J. Clin. Invest.* 99 (1997) 2446–2451.
- [12] G.J.R. Zaman, E.M. Conway, The elusive factor Xa receptor: failure to detect transcripts that correspond to the published sequence of EPR1, *Blood* 96 (2000) 145–148.
- [13] E. Camerer, W. Huang, S.R. Coughlin, Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 5255–5260.
- [14] W. Ruf, A. Dorfleutner, M. Riewald, Specificity of coagulation factor signaling, *J. Thromb. Haemost.* 1 (2003) 1495–1503.
- [15] L.V. Rao, U.R. Pendurthi, Tissue factor–factor VIIa signaling, *Arterioscler., Thromb., Vasc. Biol.* 25 (2005) 47–56.
- [16] D. Billy, H. Speijer, G. Willems, H.C. Hemker, T. Lindhout, Prothrombin activation by prothrombinase in a tubular flow reactor, *J. Biol. Chem.* 270 (1995) 1029–1034.
- [17] M.P. Esnouf, P.H. Lloyd, J. Jesty, A method for the simultaneous isolation of factor Xa and prothrombin from bovine plasma, *Biochem. J.* 131 (1973) 781.
- [18] K. Fujikawa, M.E. Legaz, E.W. Davie, Bovine factors X1 and X2 (Stuart factor). Isolation and characterization, *Biochemistry* 11 (1972) 4892–4899.
- [19] T. Morita, C.M. Jackson, Preparation and properties of derivatives of bovine factor Xa and factor Xa from which the g-carboxyglutamic acid containing domain has been removed, *J. Biol. Chem.* 261 (1986) 4015.
- [20] J. Rosing, R.F.A. Zwaal, G. Tans, Formation of meizothrombin as intermediate in factor Xa-catalyzed prothrombin activation, *J. Biol. Chem.* 261 (1986) 4224.
- [21] E. Thaler, G. Schmer, A simple two-step isolation procedure for human and bovine antithrombin II/III (heparin cofactor): a comparison of two methods, *Br. J. Haematol.* 31 (1975) 233.
- [22] R.M.A. Van Gorp, J.L.V. Broers, C.P.M. Reutelingsperger, G. Hornstra, M.C.E. van Dam-Mieras, J.W.M. Heemskerk, Peroxide-induced membrane blebbing in human endothelial cells associated with glutathione oxidation but not apoptosis, *Am. J. Physiol.* 277 (1999) C20–C28.
- [23] M.A. Van Gorp, S. Heeneman, J.L.V. Broers, N.M.H.J. Bronnenberg, M.C.E. van Dam-Mieras, J.W.M. Heemskerk, Glutathione oxidation in calcium- and p38 MAPK-dependent membrane blebbing of endothelial cells, *Biochim. Biophys. Acta* 1591 (2002) 129–138.
- [24] J.W.M. Heemskerk, W.M.J. Vuist, M.A.H. Feijge, C.P.M. Reutelingsperger, T. Lindhout, Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine and procoagulant activity of adherent platelets. Evidence for regulation by protein tyrosine kinase-dependent Ca^{2+} responses, *Blood* 90 (1997) 2615–2625.
- [25] P.E.J. Van der Meijden, M.A.H. Feijge, P.L.A. Giesen, M. Huijberts, E.P.M. van Raak, J.W.M. Heemskerk, Platelet P2Y₁₂ receptors enhance signalling towards procoagulant activity and thrombin generation: a study with healthy subjects and patients at thrombotic risk, *Thromb. Haemost.* 93 (2005) 1128–1137.
- [26] J.P. Héroult, F. Bono, C. Avril, P. Schaeffer, J.M. Herbert, Activation of human vascular endothelial cells by factor Xa: effect of specific inhibitors, *Biochem. Pharmacol.* 57 (1999) 603–610.
- [27] B.E. Maryanoff, R.J. Santulli, D.F. McComsey, W.J. Hoekstra, K. Hoe, C.E. Smith, M. Addo, A.L. Darrow, P. Andrade-Gordon, Protease-activated receptor-2 (PAR-2): structure-function study of receptor activation by diverse peptides related to tethered-ligand epitopes, *Arch. Biochem. Biophys.* 386 (2001) 195–204.
- [28] S. Banzet, N. Koulmann, N. Simler, O. Birot, H. Sanchez, R. Chapot, A. Peinnequin, X. Bigard, Fibre-type specificity of interleukin-6 gene transcription during muscle contraction in rat: association with calcineurin activity, *J. Physiol.* 566 (2005) 839–847.
- [29] J. Pieters, T. Lindhout, H.C. Hemker, In situ-generated thrombin is the only enzyme that effectively activates factor VIII and factor V in thromboplastin-activated plasma, *Blood* 74 (1989) 1021–1024.
- [30] E. Camerer, J.A. Rottingen, J.G. Iversen, H. Prydz, Coagulation factors VII and X induce Ca^{2+} oscillations in Madin-Darby canine kidney cells only when proteolytically active, *J. Biol. Chem.* 271 (1996) 29034–29042.
- [31] K. McLean, S. Schrim, A. Johns, J. Morser, D.R. Light, FXa-induced responses in vascular cells are PAR-mediated and inhibited by ZK-807834, *Thromb. Res.* 103 (2001) 281–297.
- [32] M. Riewald, V.V. Kravchenko, R.J. Petrovan, P.J. O'Brien, L.F. Brass, R.J. Ulevitch, W. Ruf, Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1, *Blood* 97 (2001) 3109–3116.
- [33] V.M. Shpacovitch, T. Brzoska, J. Buddenkotte, C. Stroh, C.P. Sommerhoff, J.C. Ansel, K. Schulze-Osthoff, N.W. Bunnett, T.A. Luger, M. Steinhoff, Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells, *J. Invest. Dermatol.* 118 (2002) 380–385.
- [34] J.R. Weinstein, S. Hong, J.D. Kulman, C. Bishop, J. Kuniyoshi, H. Andersen, B.R. Ransom, U.K. Hanish, T. Möller, Unraveling thrombin's true microglia-activating potential: markedly disparate profiles of pharmaceutical-grade and commercial-grade thrombin preparations, *J. Neurochem.* 95 (2005) 1177–1187.
- [35] P.J. Souter, S. Thomas, A.R. Hubbard, S. Poole, J. Römisch, E. Gray, Antithrombin inhibits lipopolysaccharide-induced tissue factor and interleukin-6 production by mononuclear cells, human umbilical vein endothelial cells, and whole blood, *Crit. Care Med.* 29 (2001) 134–139.
- [36] R.E. Dolmetsch, K. Xu, R.S. Lewis, Calcium oscillations increase the efficiency and specificity of gene expression, *Nature* 392 (1998) 933–936.
- [37] M.J. Ludeman, H. Kataoka, Y. Srinivasan, N.L. Esmon, C.T. Esmon, S.R. Coughlin, PAR1 cleavage and signaling in response to activated protein C and thrombin, *J. Biol. Chem.* 280 (2005) 12128–12122.